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# The amyloid precursor protein protects PC12 cells against endoplasmic reticulum stress-induced apoptosis

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**Abstract**

Endoplasmic reticulum (ER) stress is believed to play an important role in neurodegenerative disorders such as Alzheimer's disease. In the present study, we investigated the effect of the human amyloid precursor protein (APP) on the ER stress response in PC12 cells. Tunicamycin, an inhibitor of *N*-glycosylation, rapidly induced the expression of the ER-resident chaperone Bip/grp78, a known target gene of the unfolded protein response. Prolonged treatment with tunicamycin (≥ 12 h) resulted in the activation of executioner caspases 3 and 7. Interestingly, PC12 cells overexpressing human wild-type APP (APPwt) showed increased resistance to tunicamycin-induced apoptosis compared with empty vector-transfected controls. This neuroprotective effect was significantly diminished in cells expressing the Swedish mutation of APP (KM670/671NL). Similar effects were observed when ER stress was induced with brefeldin A, an inhibitor of ER-to-Golgi protein translocation. Of note, APP-mediated neuroprotection was not associated with altered expression of Bip/grp78 or transcription factor C/EBP homologous protein-10 (CHOP/GADD153), suggesting that APP acted either downstream or independently of ER-to-nucleus signaling. Our data indicate that APP plays an important physiological role in protecting neurons from the consequences of prolonged ER stress, and that APP mutations associated with familial Alzheimer's disease may impair this protective activity.

**Keywords:** brefeldin A, caspases, endoplasmic reticulum, molecular chaperone, tunicamycin, unfolded protein response.

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Endoplasmic reticulum (ER) stress triggers the accumulation of malfolded proteins within the secretory pathway and activates a conserved cellular stress response pathway, the unfolded protein response (UPR) (Kozutsumi *et al*. 1988; Kaufman 1999). In recent years, significant progress has been achieved in elucidating the molecular mechanisms of the mammalian UPR. Activation of the UPR involves the concerted action of three proximal ER transmembrane signal transducers: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and the protein kinase/endoribonuclease IRE1 (Tirasophon *et al*. 1998; Yoshida *et al*. 1998; Harding *et al*. 1999). The UPR is associated with a general inhibition of protein translation, while the expression of genes encoding molecular chaperones, such as *BiP/grp78* is simultaneously activated, thus alleviating ER stress. The UPR also triggers the removal of misfolded proteins by targeting them to the proteasome in a process known as ER-associated protein degradation (ERAD) (Friedlander *et al*. 2000). Despite these stress defense mechanisms, persisting ER dysfunction will finally lead to irreversible cell injury and apoptotic cell death (Kaufman 1999).

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*Abbreviations used*: Ac-DEVD-AMC, acetyl-DEVD-7-amido-4-methylcoumarin; APP, amyloid precursor protein; CHAPS, 3-(3-cholamidopropyldimethylammonio)-1-propane sulfonate; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FAD, familial Alzheimer's disease; PI, propidium iodide; SDS, sodium dodecyl sulfate; UPR, unfolded protein response; wt, wild type.

  

There is increasing evidence that disturbance of ER function in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease renders neurons more susceptible to cell death (Paschen and Frandsen 2001; Sherman and Goldberg 2001; Glazner and Fernyhough 2002; Ryu *et al*. 2002). It is well established that mutations in the amyloid precursor protein (APP) and presenilin-1 and -2 genes are associated with the onset of familial Alzheimer's disease (FAD; Selkoe 2001). Attenuation of the UPR has been proposed as an underlying mechanism of neurodegeneration in FAD (Katayama *et al*. 2001). Although the UPR is functional in the absence of presenilins (Sato *et al*. 2000), evidence has evolved that presenilins may be modulators of this pathway, and are required for a fully fledged ER stress response. Expression of dominant negative FAD-associated presenilin mutants was shown to downmodulate UPR target gene expression (Katayama *et al*. 1999; Imaizumi *et al*. 2001) and attenuation of translation after induction of ER stress (Yasuda *et al*. 2002). In line with this evidence, mutant presenilin 1 was suggested to increase the vulnerability to ER stress-triggered cell death (Terro *et al*. 2002). Despite the existing experimental data regarding presenilins, the role of APP in regulation of the ER stress response has not been elucidated so far. In the present study, we investigated the effect of the human APP on cell death triggered by ER stress in PC12 cells. Our data indicate that wild-type APP significantly decreases the cellular susceptibility to ER stress-induced apoptosis. This neuroprotective role of APP was compromised by the Swedish mutation of APP. Moreover, APP-mediated protection from ER stress was not associated with alterations of the UPR, indicating that APP exerts its neuroprotective effect downstream or independent of ER-to-nucleus signaling.

## Materials and methods

### Materials

Tunicamycin and brefeldin A were purchased from Alexis (Grünberg, Germany). Acetyl-DEVD-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was from Bachem (Heidelberg, Germany). All other biochemicals and chemicals came in analytical grade purity from Roche Diagnostics (Mannheim, Germany) or Roth (Karlsruhe, Germany).

### Cell culture and transfection

PC12 cell lines expressing equal amounts of human wild-type APP (PC12 APPwt, clones M5 and N10) or the Swedish double mutant KM670/671NL (Haass *et al*. 1995)(PC12 APPsw, clones Q7 and Q8), as well as vector-transfected control cells (PC12 neo, clones O1 and O3) have been described previously (Eckert *et al*. 2001). Cell lines were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 5% horse serum, 50 U/mL penicillin and 50 μg/mL streptomycin. For transient transfections, plasmids encoding enhanced green fluorescent protein (EGFP) or farnesylated GFP (GFP-F)/(BD Biosciences, Heidelberg, Germany) and Metafectene (Biontex, Munich, Germany) were diluted at a 1 : 4 ratio (μg/μL) in OptiMEM medium (Invitrogen Life Technologies, Karlsruhe, Germany) under serum-free conditions and incubated at room temperature for 30 min. The DNA-Metafectene-OptiMEM transfection mixture was then added to the culture supernatant after which cells were incubated for 24 h at 37°C and 5% $CO_2$.

### Characterization of APP-overexpressing cell lines

Cell lysates of PC12 cell lines were prepared in lysis buffer [50 mM Tris/HCl, pH 7.6, 150 mM sodium chloride, 2 mM EDTA, 1% Nonidet P-40, 2 mM dithiothreitol (DTT)], supplemented with a protease inhibitor cocktail (complete™, Roche Diagnostics, Mannheim, Germany). The lysate was centrifuged at 14 000 × *g* for 10 min and separated on an 8% sodium dodecyl sulfate (SDS)–polyacrylamide gel. The gel was blotted onto a nitrocellulose membrane and human APP was detected with polyclonal antiserum 5313 recognizing only human APP and enhanced chemiluminescence (ECL; Amersham Biosciences, Braunschweig, Germany). For quantitative detection of Aβ peptides, conditioned media of PC12 cell clones were analyzed for Aβ1–40 production using a sandwich ELISA. Cells were plated at equal density in six-well plates. After reaching confluence, 2 mL of conditioned media were collected for 18 h. Media were centrifuged to remove cell fragments, and aliquots were then used to determine Aβ(1–40). A highly specific sandwich ELISA employing monoclonal antibodies specific for the detection of Aβ(1–40) was used according to previously published protocols (Suzuki *et al*. 1994; Steiner *et al*. 1998).

### Induction of ER stress

ER stress was induced by exposure to tunicamycin and brefeldin A. Tunicamycin is a nucleoside antibiotic that inhibits *N*-glycosylation of target asparagine residues in the lumenal domains of proteins, whereas brefeldin A blocks the translocation of proteins from the ER to the Golgi apparatus by causing disassembly of the Golgi complex. Controls were treated with vehicle (dimethyl sulfoxide, 0.1%).

### Measurement of caspase-3-like protease activity

Cells were lysed in 200 μL lysis buffer [10 mM HEPES, pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 5 μg/mL aprotinin, 0.5% 3-(3-cholamidopropyldimethylammonio)-1-propane sulfonate (CHAPS)]. Fifty microliters of this lysate was added to 150 μL reaction buffer (25 mM HEPES, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 3 mM DTT, pH 7.5) and 10 μM of the fluorigenic substrate Ac-DEVD-AMC. This substrate is cleaved by caspase 3, but also by caspases 6, 7, 8 and 10 (Garcia-Calvo *et al*. 1999). Accumulation of Acetyl-DEVD-7-amido-4-methylcoumarin (AMC) fluorescence was monitored over 1 h using an Htsoft (HTS) fluorescent plate reader (excitation 380 nm, emission 465 nm). Fluorescence of blanks containing no cell lysate was subtracted from the values. Protein content was determined using the Pierce Coomassie Plus Protein Assay reagent (KMF, Cologne, Germany). Caspase activity is expressed as change in fluorescent units per microgram protein and per hour.

### Simultaneous quantification of apoptosis and necrosis

Chromatin condensation and fragmentation, which are nuclear hallmarks of apoptosis, were visualized using the DNA-binding

fluorescent dye Hoechst 33258 (Sigma, Deisenhofen, Germany) while necrosis was evaluated by propidium iodide (PI) uptake. PI is a DNA binding dye which enters dead cells through damaged membranes, hence indicating primary as well as secondary necrosis. Cells cultured on 24-well plates were treated with tunicamycin or vehicle and stained live with Hoechst 33258 and PI at final concentrations of 1 μg/mL and 2 μg/mL, respectively. After incubation for at least 10 min, nuclear morphology and PI uptake was observed using an Eclipse TE 300 inverted microscope (Nikon, Düsseldorf, Germany) and a 20 × dry immersion objective with the following optics: excitation, 340–380 nm; dichroic mirror, 400 nm; emission, 435–485 nm for Hoechst and excitation, 540–580 nm; dichroic mirror, 595 nm; emission, 600–660 nm for PI. Digital images were acquired with a SPOT-2 camera using SPOT software version 2.2.1 (Diagnostic Instruments, Sterling Heights, MI, USA). For each time point and treatment, a total number of 300 cells were analyzed for apoptotic morphology and PI uptake in three subfields of each culture. All experiments were performed at least three times with similar results.

### Semiquantitative RT-PCR

One microgram of total RNA was used in an RT-reaction together with 2.5 μg random hexamers and dNTPs at a final concentration of 0.5 mM. After heating to 65°C for 5 min, the mixtures were chilled on ice and supplemented with reaction buffer, DTT (final concentration 10 mM), and 200 units Moloney murine leukemia virus-reverse transcriptase (Invitrogen Life Technologies, Karlsruhe, Germany). First strand cDNA synthesis was carried out at 42°C for 50 min Reactions were stopped by incubation at 70°C for 15 min cDNAs were subsequently analyzed for expression of *BiP/grp78* and *CHOP/GADD153*, with *GAPDH* serving as internal control. PCR reactions were performed with Taq polymerase (Eppendorf, Hamburg, Germany) according to the instructions of the manufacturer. A 476-bp product of *BiP/grp78* was amplified with primers 5′-GGTACATTTGATCTGACTG-3′ and 5′-CACTTCCATAGAGTTTGCTG-3′, and a 441-bp product of *CHOP/GADD153* with primers 5′-CTTCACTACTCTTGACCCTGCAT-3′ and 5′-ATGTGCACTGGAGATTACTGCTT-3′. A 355-bp product of *GAPDH* was amplified with primers 5′-CTCGTGGTTCACACCCAT-3′ and 5′-GGCTGCCTTCTCTTGTGA-3′ in combination with either BiP/grp78 or CHOP/GADD153 primers. For *BiP/grp78* and *GAPDH*, PCR was carried out in 25 PCR cycles with 1 min denaturation at 95°C, 1 min annealing at 51°C, and 1 min elongation at 72°C. For *CHOP/GADD153*, PCR was carried out in two initial PCR cycles with 1 min denaturation at 95°C, 1 min annealing at 51°C, and 1 min elongation at 72°C, after which the GAPDH primers were added and 25 additional cycles were performed. Initial experiments were carried out to ascertain that PCR was within the linear amplification range for all three investigated genes. PCR products were separated by agarose gel electrophoresis (1.5%) and visualized with 0.1% ethidium bromide under UV transillumination using a CCD camera-based gel documentation system (MWG, Ebersberg, Germany). Levels of *BiP/grp78* and *CHOP/GADD153* expression relative to *GAPDH* were quantified densitometrically using ONEDScan software (Scanalytics, Fairfax, VA, USA).

### Western blot

Thirty micrograms of whole cell lysate (lysis buffer: 68.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, and protease inhibitors) was loaded onto a 15% SDS–polyacrylamide gel. Proteins were separated for 1 h at 120 V and then blotted to nitrocellulose membranes (Protean BA 83; 2 μm; Schleicher & Schuell, Dassel, Germany) in Towbin-buffer [25 mM Tris, 192 mM glycine, 20% methanol (v/v) and 0.01% SDS] at 15 V for 45 min. The blots were blocked in blocking buffer (5% non-fat milk, 15 mM Tris-HCl pH 7.5, 200 mM NaCl and 0.1% Tween-20) at room temperature for 2 h. Membranes were incubated with a rabbit polyclonal anti-active p20 caspase 7 antiserum (Cell Signaling, New England Biolabs, Frankfurt, Germany, 1 : 1000 dilution), a rabbit polyclonal anti-active p20 caspase 3 antiserum (Merck-Frosst, Kirkland, Canada, 1 : 1000 dilution) and a mouse monoclonal anti-KDEL antibody raised against the synthetic peptide SEKDEL based on the rat BiP/grp78 sequence (Stressgen, Victoria, Canada, 1 : 1000 dilution). To confirm equal loading of samples, a mouse monoclonal anti-α-tubulin antibody (clone DM 1 A; 1 : 5000, Sigma) or a mouse monoclonal anti-JNK1 antibody (clone 333, 1 : 1000, Pharmingen Becton Dickinson, Hamburg, Germany) were used. Antibodies were diluted in blocking solution and blots were incubated overnight at 4°C. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Promega, Heidelberg, Germany) used 1 : 5000 in blocking solution. Blots were developed using the Pierce SuperSignal substrate chemiluminescence reagent.

### Statistics

Data are given as means ± SEM. For statistical comparison, *t*-test or one-way ANOVA followed by Tukey's test were employed. *p*-values smaller than 0.05 were considered statistically significant.

## Results

### Tunicamycin induces apoptosis in PC12 cells

Tunicamycin blocks *N*-glycosylation of proteins, thus leading to accumulation of hypoglycosylated proteins in the ER lumen and disturbance of the secretory pathway. Prolonged exposure to tunicamycin has been previously shown to induce apoptosis in rat PC12 cells (Nakagawa and Yuan 2000). To establish optimal working concentrations and incubation periods for tunicamycin-induced apoptosis, we performed initial DEVDase assays in parental PC12 cells. Cleavage of fluorigenic substrate Ac-DEVD-AMC by cytosolic protein extracts indicates the presence of caspase 3-like protease activity in the cultures. Exposure to tunicamycin for 20 h induced DEVD cleavage in a dose-dependent manner (Fig. 1a). A significant increase in DEVD cleavage activity was observed at a concentration of 0.3 μM, and more pronounced at a concentration of 1 μM and 3 μM for tunicamycin (Fig. 1a). Time course studies revealed the onset of DEVD cleavage activity to occur 12 h after treatment with 1 μM tunicamycin (Fig. 1b). Activation of executioner caspases was also confirmed by western blotting with antibodies selectively recognizing the cleaved (activated) forms of executioner caspases 3 and 7 (Fig. 1c). Tunicamycin induced prominent activation of both caspases in PC12 neo O1 cells after 20 h of treatment.

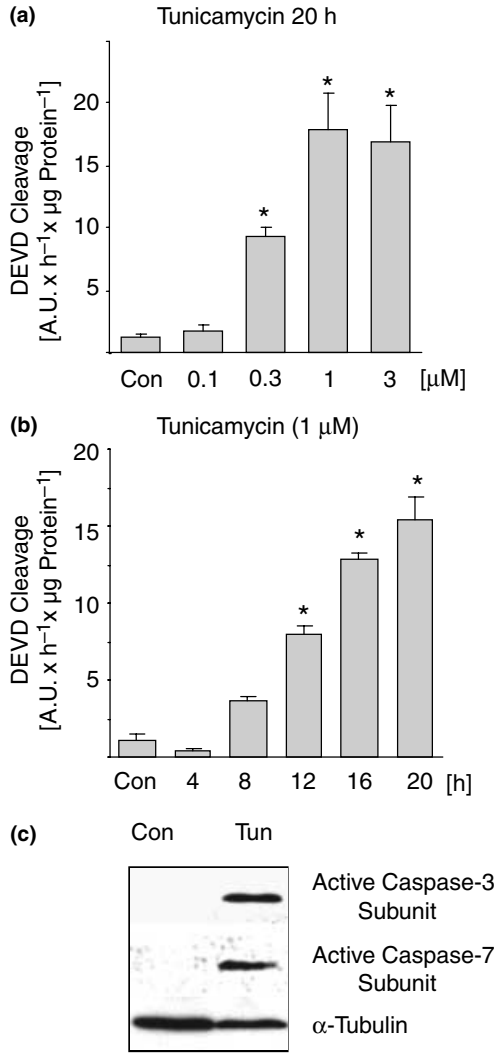


**Fig. 1** ER stressor tunicamycin induces apoptosis in a time- and dose-dependent manner in rat PC12 cells. Cultures were treated for 20 h with the indicated concentrations of tunicamycin or vehicle (Con) (a). Alternatively, cultures were treated with 1 µM tunicamycin for the indicated time periods (b). Controls (Con) were exposed to vehicle for 20 h. Cleavage of fluorigenic Ac-DEVD-AMC by cytosolic extracts was monitored over 1 h using a fluorescent plate reader. Data are means ± SEM from $n = 4$–6 cultures per treatment. Experiments were repeated two times with similar results. AU = arbitrary fluorescence units. *$p < 0.05$: Difference from vehicle-treated controls. (c) Caspases 3 and 7 are activated during ER stress-mediated apoptosis. PC12 neo O1 cells were treated with 1 µM tunicamycin (Tun) or vehicle (Con) for 20 h. Active caspase 3 and caspase 7 subunits were detected by western blotting with α-tubulin serving as a loading control.

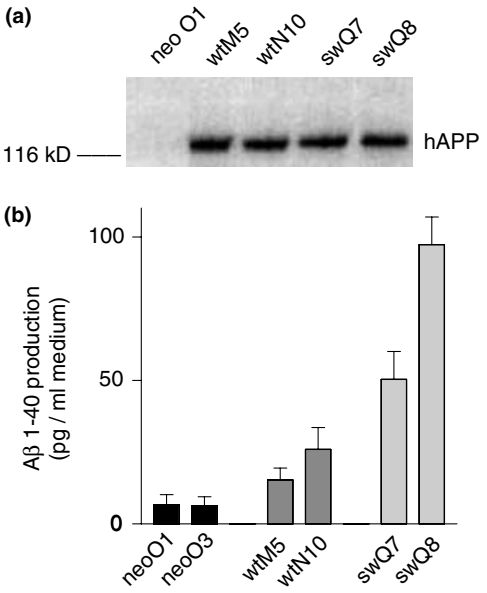


**Fig. 2** Characterization of APP-overexpressing PC12 cell clones. Expression levels of human APP in cell lines PC12 APPwt M5, PC12 APPwt N10, PC12 APPsw Q7 and PC12 APPsw Q8 was investigated by western blotting with the 5313 anti-APP antiserum recognizing only human APP. PC12 neo O1 cells served as a control (a). Quantitative detection of Aβ 1–40 in the culture supernatants of PC12 neo O1, PC12 neo O3, PC12 APPwt M5, PC12 APPwt N10, PC12 APPsw Q7 and PC12 APPsw Q8 cells was performed by sandwich ELISA analysis employing monoclonal antibodies specific for the detection of Aβ(1–40) (b). Data are means ± SEM from three independent experiments per clone.

## Characterization of cells overexpressing APPwt or APPsw

To investigate the effect of APP overexpression on ER stress-induced apoptosis, we used PC12 APPwt cells stably overexpressing wild-type human APP, PC12 APPsw cells stably transfected with APP KM670/671NL, the Swedish double mutant of APP, and PC12 neo cells (clones O1 and O3) as vector-transfected controls (Eckert *et al*. 2001). Expression of human APP in the investigated cell lines was confirmed by western blotting (Fig. 2a). Equal expression levels of APP were detectable in clones transfected either with human wild-type APP (clones M5 and N10) or with the Swedish double mutation form of APP (clones Q7 and Q8). Culture supernatants of the APP-overexpressing clones were also analyzed for Aβ1–40 production (Fig. 2b), revealing elevated Aβ1–40 levels, particularly in the APPsw-overexpressing clones Q7 and Q8.

## Overexpression of APPwt protects PC12 cells from apoptosis induced by tunicamycin with less protection exerted by APPsw

Subsequently, we investigated the effect of APP overexpression on tunicamycin-induced apoptosis. Overexpression of APPwt and APPsw did not affect basal caspase3-like activity in the vehicle-treated cultures (Fig. 3a). However, overexpression of APPwt in cell lines M5 and N10 significantly reduced effector caspase activation after treatment with tunicamycin (1 µM and 3 µM) for 20 h compared with

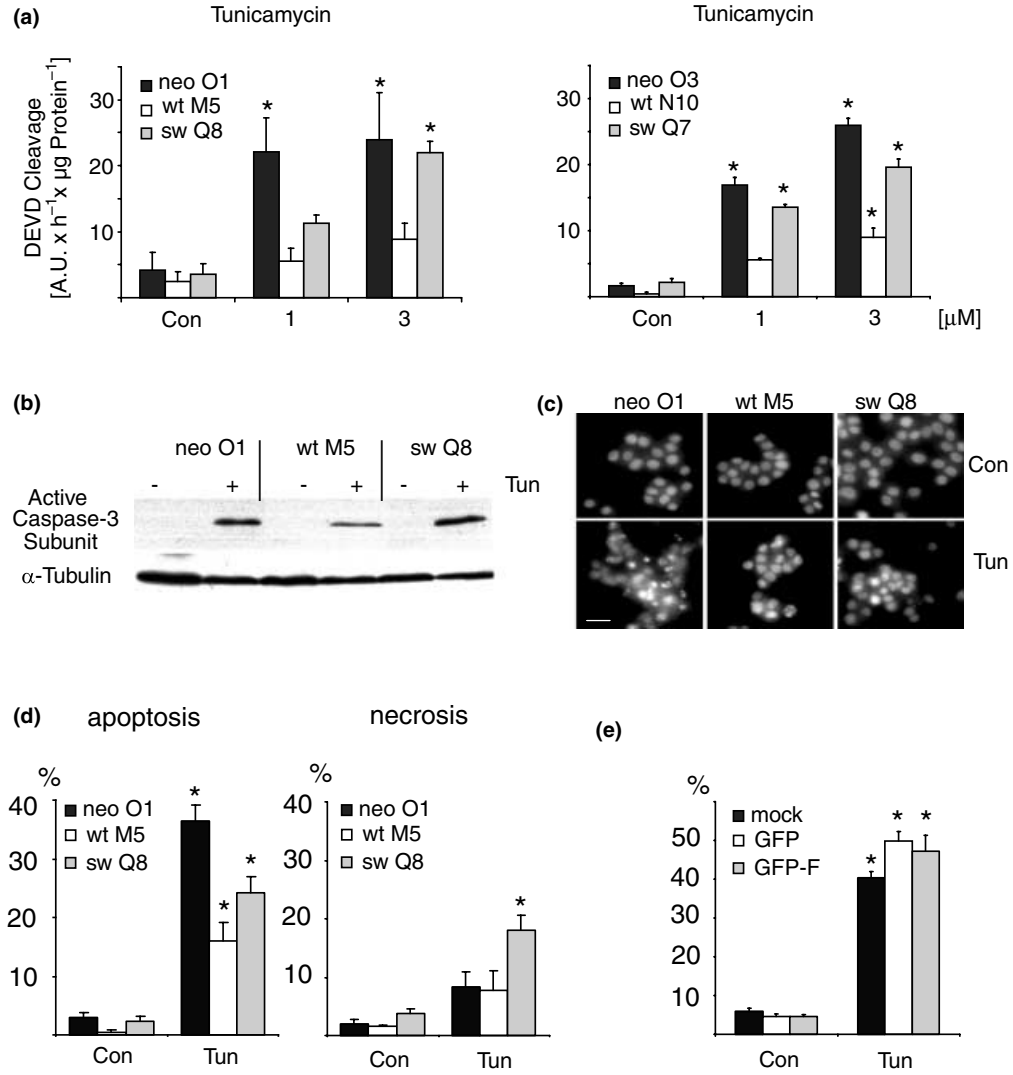


**Fig. 3** Overexpression of wild-type APP protects PC12 cells from tunicamycin-induced apoptosis. (a) PC12 neo O1 (neo), PC12 APPwt M5 (wt) and PC12 APPsw Q8 (sw) cells were treated with 1 and 3 μM tunicamycin for 20 h (left panel). PC12 neo O3 (neo), PC12 APPwt N10 (wt) and PC12 APPsw Q7 (sw) cells were treated with 1 and 3 μM tunicamycin for 20 h (right panel). Control cultures were treated with vehicle (Con). Caspase 3-like activity was monitored over one hour. Data are means ± SEM from $n = 6$ cultures per treatment. Experiments were repeated two times with similar results. AU = arbitrary fluorescence units. *$p < 0.05$: Difference from vehicle-treated control (PC12 neo, clone O1 or O3). In comparison with PC12 neo treated with tunicamycin, protection from tunicamycin-induced caspase activation in both PC12 APPwt clones was statistically significant at both 1 and 3 μM. (b) Activation of executioner caspase 3 during tunicamycin-induced apoptosis. PC12 neo O1, PC12 APPwt M5 and PC12 APPsw cells were treated with 1 μM tunicamycin (Tun) or vehicle (Con) for 20 h. Active caspase 3 p20 subunits were detected by western blotting with α-tubulin serving as a loading control. (c) Nuclear apoptosis visualized by Hoechst staining. Cultures of PC12 neo O1 (neo), PC12 APPwt M5 (wt) and PC12 APPsw (sw) cells were treated with 1 μM tunicamycin (Tun) or vehicle (Con) for 20 h. Scale bar: 25 μm. (d) Parallel quantification of apoptosis and (primary and secondary) necrosis after induction of ER stress with tunicamycin. PC12 neo O1 (neo), PC12 APPwt M5 (wt) or PC12 APPsw (sw) cells were exposed to 1 μM tunicamycin or vehicle for 20 h. The percentage of apoptotic and necrotic cells in the different cultures is shown. Data are means ± SEM from $n = 4$ cultures per treatment. Experiments were repeated two times with similar results. *$p < 0.05$: Difference from vehicle-treated controls. In comparison with PC12 neo treated with tunicamycin, protection from tunicamycin-induced apoptosis in PC12 APPwt cells was statistically significant. (e) Overexpression of membrane-bound EGFP does not affect ER stress-triggered cell death in PC12 cells. PC12 neo O1 were transfected with EGFP, EGFP-F or mock transfected. Twenty-four hours after transfection, cells were treated with 1 μM tunicamycin (Tun) or vehicle (Con). After an additional 20 h, the nuclear morphology of cells expressing EGFP or EGFP-F was analyzed by Hoechst staining and fluorescence microscopy. For each culture, 100 individual cells were analyzed. Data are means ± SEM from $n = 6$ cultures per treatment. *$p < 0.05$: Difference from vehicle-treated controls.

vector-transfected control cell lines PC12 neo O1 and PC12 neo O3 (Fig. 3a, left and right panels). In contrast to PC12 APPwt, the reduction of tunicamycin-triggered DEVDase activity was much less prominent in PC12 APPsw cells. Western blotting revealed only weak activation of caspase 3 in lysates of PC12 APPwt cells treated with 1 μM tunicamycin for 20 h, whereas activation was more pronounced in PC12 neo and PC12 APPsw cultures (Fig. 3b). Nuclear Hoechst staining showed that a significant number of nuclei were condensed and/or fragmented in tunicamycin-treated PC12 neo and PC12 APPsw cultures (Fig. 3c). In contrast, most of the nuclei of tunicamycin-treated PC12 APPwt cells did not reveal an apoptotic morphology. In addition to the observed effects on apoptotic cell death, APP might also have affected the appearance of necrosis in the cultures. Hence, we quantitatively determined the rate of apoptosis and necrosis in tunicamycin-treated cultures by simultaneous staining of live cells with Hoechst and the membrane-impermeable dye PI. Membrane leakage is a hallmark of necrosis, but is also an endpoint of neuronal apoptosis *in vitro* (secondary necrosis). In concordance with the DEVDase assays, overexpression of APPwt significantly reduced the percentage of apoptotic cells after treatment with 1 μM tunicamycin compared with vector-transfected controls (Fig. 3d, left panel). Overexpression of APPsw also led to a limited protection against 1 μM tunicamycin-induced apoptosis, but at the same time significantly increased necrosis in the cultures (Fig. 3d, right panel). APPsw-triggered necrosis was also observed in the dimethyl sulfoxide-treated controls, although it was not statistically significant. These data suggest that APPsw shifts the mode of ER stress-induced cell death from apoptosis to necrosis. We further confirmed the protective role of APP in additional transient transfection experiments. To rule out non-specific effects of overexpressed membrane proteins such as APP on cell death triggered by ER stress, we transfected PC12 neo cells with enhanced green fluorescent protein (EGFP) and EGFP-F, a membrane-bound form of GFP and analyzed the fate of transfected cells after treatment with tunicamycin on the single cell level. GFP-F carries the farnesylation motif of H-Ras, a protein that is transported to the cell membrane via the ER/Golgi pathway (Silvius 2002). In comparison with GFP-transfected cells, there was no significant effect of GFP-F on cell death triggered by tunicamycin (Fig. 3e).

### APPwt protects PC12 cells from apoptosis induced by a second ER stressor, brefeldin A

In addition to tunicamycin, we also investigated the effect of wild-type and mutant APP on cell death induced by a second ER stressor, brefeldin A. Brefeldin A induces disassembly of the Golgi complex which leads to a blockade of protein translocation to the Golgi apparatus and accumulation of proteins in the ER. Analysis of the time course of DEVDase activation in response to brefeldin A revealed significant

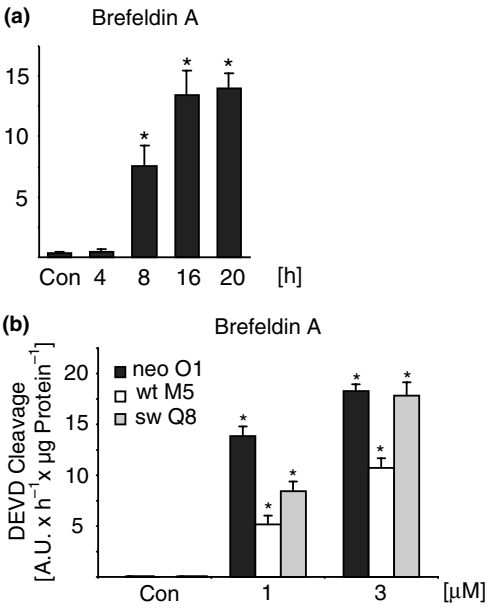


**Fig. 4** Overexpression of wild-type APP protects PC12 cells from brefeldin A-induced apoptosis. (a) Brefeldin A induces apoptosis in a time-dependent manner in rat PC12 cells. Cultures were treated for 4, 8, 16 and 20 h with 1 μM brefeldin A or vehicle (Con). (b) Cultures of PC12 neo O1 (neo), PC12 APPwt M5 (wt) and PC12 APPsw Q8 (sw) cells were treated with 1 μM brefeldin A or vehicle (Con) for 20 h. Cleavage of fluorigenic Ac-DEVD-AMC by cytosolic extracts was monitored over one hour using a fluorescent plate reader. Data are means ± SEM from $n = 4$–6 cultures per treatment. Experiments were repeated two times with similar results. AU = arbitrary fluorescence units. *$p < 0.05$: Difference from vehicle-treated controls. In comparison with PC12 neo treated with brefeldin A, protection from brefeldin A-induced DEVDase activity in PC12 APPwt cultures was statistically significant at both 1 and 3 μM.

DEVDase activity 8 h after start of treatment (Fig. 4a). As observed for tunicamycin, APPwt overexpression significantly increased resistance to apoptosis induced by treatment with 1 and 3 μM brefeldin A for 20 h (Fig. 4b).

### Overexpressed APP does not affect induction of the UPR

In light of the proposed role of presenilins in regulation of the UPR (Katayama *et al*. 1999) and the fact that APP processing requires presenilins, we investigated whether overexpression of APP was associated with alterations in ER stress-induced activation of UPR target genes, such as the anti-apoptotic molecular chaperone *BiP/grp78* (Kozutsumi *et al*. 1988). In initial experiments, analysis of *BiP/grp78* expression after induction of ER stress was performed by western blotting. BiP/grp78 protein levels after treatment with 1 μM tunicamycin for 8 h were significantly up-regulated in PC12 neo, PC12 APPwt and PC12 APPsw cultures, indicating that the UPR was functional in the APP-overexpressing cell lines (Fig. 5a). For a more detailed

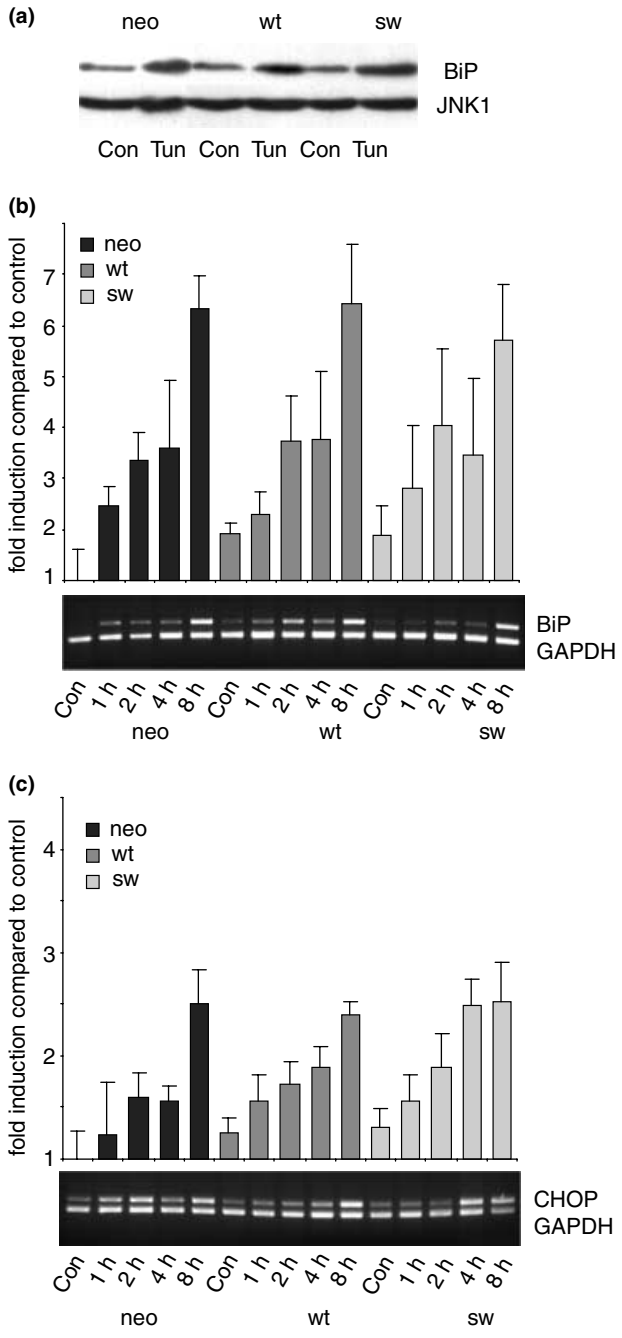


**Fig. 5** The kinetics and magnitude of UPR target gene induction are not significantly affected by overexpression of APP. Analysis of BiP/grp78 protein levels after induction of ER stress with tunicamycin (a). PC12 neo O1 (neo), PC12 APPwt M5 (wt) and PC12 APPsw Q8 (sw) cells were treated with 1 μM tunicamycin for 8 h and subjected to western blotting with an anti-BiP/grp78 antibody. JNK1 served as a loading control. Semiquantitative RT-PCR analysis of *BiP/grp78* (b) and *CHOP/GADD153* (c) expression levels after induction of ER stress with tunicamycin. cDNA from PC12 neo O1 (neo), PC12 APPwt M5 (wt) and PC12 APPsw (sw) cells treated with 1 μM tunicamycin for 1, 2, 4 and 8 h was amplified by 25 cycles (*BiP/grp78*) or 27 cycles (*CHOP/GADD153*) of PCR with *GAPDH* (25 cycles) serving as internal control. Control cultures (Con) were treated with vehicle for 8 h. Reaction products were separated by agarose gel electrophoresis and visualized with 0.1% ethidium bromide. Quantification of BiP/grp78 and CHOP/GADD153 mRNA levels relative to GAPDH was performed with the ONEDScan gel analysis software. Data are means from $n = 4$ cultures per treatment.

evaluation of the effect of APP on the UPR we performed semiquantitative RT-PCR analysis of *BiP/grp78* induction, as well as expression of a second UPR target gene, the pro-apoptotic transcription factor *CHOP/GADD153* (Wang *et al.* 1996). To analyze the kinetics and magnitude of tunicamycin-induced expression of both genes, we treated all three cell lines with 1 μM tunicamycin for 1, 2, 4 and 8 h. Activation of the UPR by tunicamycin was a rapid event as both target genes were detectably already up-regulated after 1 h with a steady increase in transcript levels over the entire time course analyzed (Fig. 5b,c). Densitometric analysis revealed that the kinetics and magnitude of tunicamycin-triggered *BiP/grp78* and *CHOP/GADD153* induction were very similar for all three investigated cell lines, thus indicating that APPwt and APPsw overexpression did not detectably affect induction of the UPR.

## Discussion

Here we report that wild-type APP significantly protects PC12 cells from ER stress-induced neuronal apoptosis. Our data therefore indicate that APP may play an important physiological role in protecting neurons from the consequences of prolonged malfunctions in protein quality control.

APP has been shown to protect neuroblastoma cells against UV-mediated apoptosis by controlling p53 activation at the post-translational level (Greenfield *et al.* 1999). However, ER stress-triggered cell death has been reported to occur in a p53-independent fashion (Shao *et al.* 1996), thus suggesting that APP inhibits ER stress-mediated cell death by a molecular mechanism distinct from that observed in protection from UV-induced apoptosis. The major processing pathway of wild-type APP involves its proteolytic cleavage by α-secretase and results in the release of soluble, nonamyloidogenic sAPPα. It has been reported that sAPPα may exert a neuroprotective effect by activation of the nuclear factor-kappa B signaling pathway (Barger and Mattson 1996). However, our own EMSA analyses did not reveal APP-dependent alterations of nuclear factor-kappa B activity after induction of ER stress in the neo-transfected and APP-overexpressing PC12 cell lines (Kögel *et al.* unpublished data) suggesting APP-mediated protection from ER stress occurs in a nuclear factor-kappa B-independent manner.

In comparison with PC12 neo controls, tunicamycin-triggered caspase activation was only moderately reduced in cells overexpressing the Swedish mutant of APP. In addition, induction of ER stress induced a significant shift from apoptosis to necrosis in the APPsw-overexpressing cultures, arguing for an impaired, or even nonexistent protection against ER stress by APPsw. As observed by ELISA analysis, processing of mutant APP was associated with significantly enhanced Aβ-levels in the supernatants of the cultures. Of note, we did not observe significant apoptosis and necrosis in untreated APPsw-overexpressing cultures, indicating that at the prevailing concentrations, Aβ might not be sufficient to induce cell death *per se*. Still, the diminished protective function of APPsw against tunicamycin-induced cell death could have been caused by a cumulative effect of Aβ overproduction and ER stress. Clearly, due to the preference for the amyloidogenic β-secretase processing pathway, expression of the Swedish mutant APPs is associated with diminished sAPPα-levels (Selkoe 2001). In contrast to sAPPα, the sAPPβ-molecule generated in the amyloidogenic pathway does not possess any neuroprotective properties (Furukawa *et al*. 1996). After processing of APP by either α- or β-secretase, ensuing cleavage of the C83 or C99 intermediates by γ-secretase leads to generation of AICD. AICD is able to form a complex with the transcriptional coactivator Fe65 and translocate to the nucleus where it acts as a transcription factor (Cao and Sudhof 2001). Interestingly, the amyloidogenic pathway has also been implicated in generation of AICD. It was proposed that C99 (generated by β-secretase cleavage) is a much better γ-secretase substrate than C83 (generated by α-secretase cleavage) (LaFerla 2002), suggesting increased AICD levels in the PC12 APPsw cultures. It was reported that AICD induces cell death upon overexpression (Kinoshita *et al*. 2002). However, the sarcoplasmic-/endoplasmic-reticulum $Ca^{2+}$-ATPase (SERCA) gene might be a direct transcriptional target of AICD (Leissring *et al*. 2002). As the activity of many ER chaperones such as calreticulin and calnexin depends on a high ER $Ca^{2+}$ content, AICD expressed at physiological levels might also enhance cellular resistance to ER stress by increasing the efficiency to cope with accumulated and misfolded proteins in the ER.

ER stress elicits a homeostatic response, the unfolded protein response (UPR) which enables cells to dispose erroneous and potentially harmful proteins. It was recently suggested that FAD-linked mutations of presenilin 1 down-modulate induction of the UPR (Katayama *et al*. 1999). However, the UPR is still present in presenilin-1 and -2-deficient cells (Sato *et al*. 2000), as well as in cells bearing dominant-negative presenilin mutations. In our experiments, overexpression of both wild-type APP and the Swedish mutant of APP did not affect the expression of the two classical UPR targets BiP/grp78 and CHOP/GADD153 under basal conditions (i.e. in the absence of ER stress). Furthermore, we did not detect baseline differences in the expression changes of other established UPR target genes such as grp94, calreticulin and protein disulfide isomerase in the APP-overexpressing PC12 cells (Kögel *et al.* unpublished data). In addition to a lack of basal expression differences of UPR target genes, APPwt and APPsw did not significantly impair the transcriptional response to ER stress, as we did not observe detectable, APP-dependent alterations in the kinetics and magnitude of BiP/grp78 and CHOP/GADD153 activation after tunicamycin-treatment. Thus, our data suggest that: (i) APP itself is not involved in regulation of the UPR and (ii) that the proposed UPR down-modulation by mutant presenilins is not causally related to APP processing or increased Aβ levels, but may represent APP-independent biological function of presenilins.

In conclusion, we found that APP potently enhances the capability of neurons to cope with ER stress and to escape from cell death. These findings could be highly relevant for Alzheimer's disease pathogenesis, as ER processing defects and protein misfolding might be early events in Alzheimer's disease.

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